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# **Software development for tracing QDs-labeled cells in coculture**



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# **Software development for tracing QDs-labeled cells in coculture**

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# Contents

1. Abstract . . . . .	5
2. Introduction . . . . .	5
3. The experiment section. . . . .	6
4. Development Assay . . . . .	7
5. Development of the software . . . . .	10
5.1. Idea of the data analyze . . . . .	10
5.2. Quantification based on the transmission . . . . .	11
5.2.1. Principle of the analysis . . . . .	11
5.2.2. Example for data analysis with identified cells . . . . .	14
5.3. Quantification based on DAPI . . . . .	18
5.3.1. Principle of the analysis . . . . .	18
5.3.2. Example for data analysis with identified nuclei . . . . .	21
5.4. Software module . . . . .	24
6. Result and discussion . . . . .	27
7. Outlook . . . . .	29
8. Acknowledgement . . . . .	30
9. References . . . . .	31

## Abbreviation and explanation of words

QDs	Quantum dots
ImagJ	Software
MathLab	Software
Igor Pro	Igor Pro <i>WaveMetrics</i>
DAPI	4', 6-Diamidino-2-phenylindole, dilactate
ROI	Region Of Interest

# 1. Abstract

The development of science allowed people to gradually realize the enormous changes from hand to machine operation. One of the most important inventions is the computer, which makes life modern and automatic. Since 1946, the date of the emergence of the first computer, it has been subject to significant progress and development, although this has begun to slow down at the beginning of the twenty-first century. Computer technology has become increasingly popular and sophisticated and is being used more and more widely in various different areas. As a result, people cannot live without computers, but it brought great convenience to them. For example: database, of the ability to make complicated calculations, quick and private communication and so on. Certainly it also makes work for researchers much easier and faster. Nowadays roughly more than half of research programs are related to computers. Microscope scanning, computer imaging, image analysis and counting of cells are all dependent on the computer. As a result of this, we also need develop and expand existing software for new technology. By modifying the source code of software so we can make the computer operate in accordance with our ideas. Thus it is important to write the software.

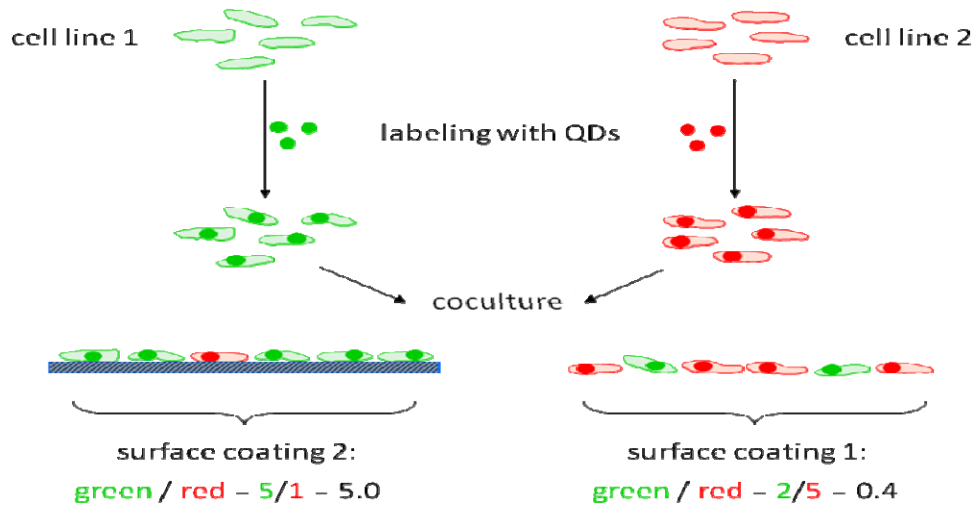
## 2. Introduction

This new computer software will be the result of our experiment. As known, tracing cells of different types in coculture is very complicated, mainly because many cells have very similar morphology and it is difficult to distinguish the cells by morphology. Currently there is no simple assay which allows such measurements, since there is no easy way to automate quantitative distinction between the individual cell types. This is the reason why we decided to find a new

method to realize automate this process. But the most important thing is to develop an assay for quantification of cell adhesion within cell-coculture, since there are obviously too many cells to count with hands and eyes. In addition to the experiment we also intend to make a statistic of the different cells. Under such conditions and requirements we will choose one piece of software which could realize the ideas of quantification of cell adhesion. We could have chosen Igor Pro, ImageJ or Matlab for our experiment. These standard image analysis programs are all capable of identifying cells as borders of cells are easily recognized in the transmission images. However we find Igor Pro[1] is more suitable than ImageJ for this experiment because many images will be analyzed at the same time. In addition Igor has better operating capabilities than Matlab and could be modified at any time, and our research is also based on Igor Pro. A more detailed explanation about Igor Pro will be given in the next chapter.

### **3. The experiment section**

Two different colors are chosen for labeling the cells which are appropriate for the filter set of the fluorescence microscope used for imaging. For our experiment the problem is how to distinguish the two different cells, thus we refer to color 1 as green and color 2 as red for our emission waves, though any two different colors are also possible. In order to improve the intensity and extend time for observation of the labeled cells we add quantum dots batches as a high concentration, but the concentration can not be so high because of the cytotoxicity[2]. After that we use the laser microscope to record the image. By calculating the ratio of the two kinds of labeled cells[3], we can realize the quantification of the cells with image analysis.



Figure[4] E-1: Cells of type 1 are labeled with QDs of green color, and cells of type 2 are labeled with QDs of red color. The two different cells are mixed and seeded in coculture afterwards on a substrate with different surface coatings. Determining the ratio of cells of each type on each surface coating is possible because they are labeled with QDs of the respective color. In the example shown, cells of type 1 adhere better to surface coating 2, whereas cells of type 2 adhere better to surface coating 1.

## 4. Development Assay

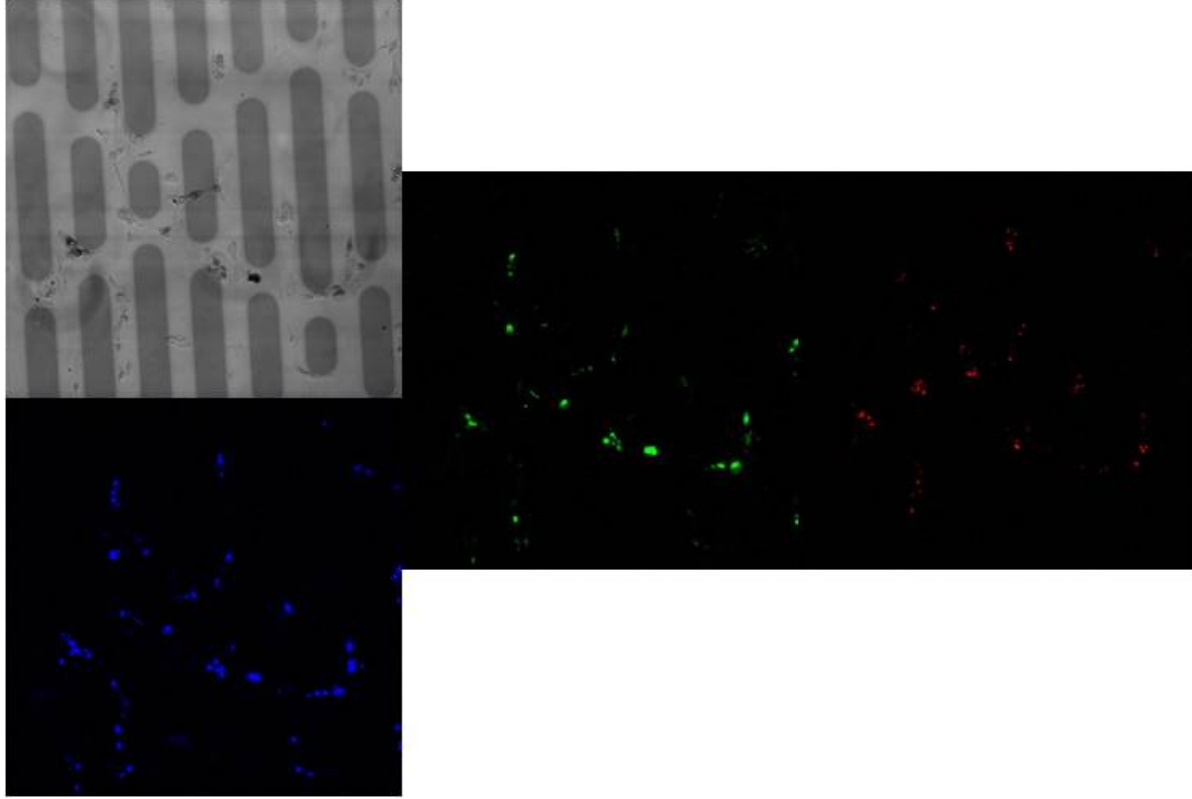
Right now the best way to analyze an image is to separate the fluorescence channels, which are quantum dots of different colors and transmission. In order to get the ratio we use the method based on the counting of the green and red quantum dots.  $N_{\text{green}}/N_{\text{red}}$  would be presented as the ratio of the different labeled cells. This method makes the borders of the cells easy to recognize and we now use the standard image analysis program Igor, which will be explained in greater detail in the section “Development of the software”. Actually a digital mask will be based on the matrix of the threshold. All the positions of the cells are presented as a mask and the rest of the image is all black. Then the digital mask will be put on the top of the image of the green and red fluorescence channel, so the fluorescence

stays only at the position where cells have been identified. This method avoids the need to count or to analyze the quantum dots directly, because they are covered by the black area. It can determine the position of each cell if there is green or red fluorescence in the corresponding fluorescence channel. The size of the single cell is important, because the size of the cells and the size of the QDs have to match correctly. However, we could still change the checking size in Igor. The problem might be resolved in this way, which is checked by identifying objects in the fluorescence channel within the location of the mask of the cell. At the same time it is also important to get rid of the background and modify the pictures from the microscope to eliminate the noise. The transmission channel in the analysis image will be described in greater detail in the next chapter.

For our experiment it is very important to analyze the cells on different substrates. The description above only applies to glass and cell culture substrates. If we put the cells on a gold surface, a problem would emerge. Under the microscope there is a scattering of light on the gold surface, which causes the lines of the gold to cast shadows. Actually the bigger problem for analysis is that Igor cannot make the digital mask of the cells, because the shadows prevent the creation of the correct mask as they disturb the substrates in the transmission channel, meaning that we cannot use the transmission channel to identify the location of the cells. We therefore use another DAPI (4', 6-Diamidino-2-phenylindole, dilactate), which is a common blue fluorescent dye used for staining of the nuclei of live cells. The blue fluorescence of the cells is then marked during imaging. It appears as a blue spot in the blue fluorescence channel. Apart from that, the method is the same as the description above. The digital mask is created from the blue-colored nuclei and the perinuclear regions and also is then overlaid with the image of the green and red fluorescence channel. So the fluorescence is checked at the same position in which nuclei and their perinuclear region have been identified. This scheme for data



analysis turned out to be suitable for determining the amount of green and red labeled cells,  $N_{\text{green}}/N_{\text{Dapi}}$  and  $N_{\text{red}}/N_{\text{Dapi}}$ , respectively. The algorithm is shown in Figure B-1 and the details will be described clearly below.



**absolute value**

**N0: (summation N trans or DAPI)**

**N1: (summation N green)**

**N2: (summation N red)**

**Ratio**

**N (green/trans or dapi) =  $N1/N0$**

**N (red/trans or dapi)=  $N2/N0$**

$$\left. \begin{aligned} N'_{\text{red}} &= \frac{N_{\text{red}}}{N_{\text{transm/DAPI}}} \\ N'_{\text{green}} &= \frac{N_{\text{green}}}{N_{\text{transm/DAPI}}} \end{aligned} \right\} \frac{N'_{\text{red}}}{N'_{\text{green}}}$$

Figure D-1: Multiplexing to calculate the total amount of labeled cells in the different channel (Transmission, green, and red) as Example.

## 5. Development of software

### 5.1. Idea of the data analyze

Because we need specialized software in order to process the large number of images in the comparative cell adhesion assay and to automate the analysis process, we came up with the first idea, which was to use the absolute fluorescence intensities  $I_{\text{green}}$  and  $I_{\text{red}}$  of the respective QD emission in both of the channels to calculate the  $I_{\text{green}}/I_{\text{red}}$  for the ratio of cells present on the analyzed substrate. But it is difficult to get accurate results, because the background intensity could affect the fluorescence of the QDs, which themselves have different levels of auto fluorescence. In addition, it is impossible to a matrix structure based on fluorescence.

The second idea was to determine the areas  $A_{\text{green}}$  and  $A_{\text{red}}$  from the green and red channel of the fluorescence image, respectively, which had fluorescence intensity over a certain threshold. This method does not allow the area of the cells to be accurately measured because the QDs may not be evenly distributed over the surface of the cell, which would result in incorrect QDs ratios.

In the end we created a new program based on Igor, which uses routine functions to recognize particles on the basis of brightness and contrast differences compared to a background and counts the number of the cells with green and red QDs labels. In addition to these functions, the filter thresholds for brightness intensities and particle areas are also necessary [4]. The main idea is that we make a digital mask which is transparent at positions where there are cells, and black at all other positions. Actually this is using the matrix of the threshold shown in the Figure D-I-1. Furthermore, using the mask we can check the labeled QDs in the cells. Specifically, we would separate the blue fluorescence channel image/ transmission channel image, green fluorescence channel image and red fluorescence channel image from the overlay of the image and can then consider green and red filled cells or filled vesicles as labeled in green and red where fluorescence can be identified within the region of the nucleus and the corresponding perinuclear region.

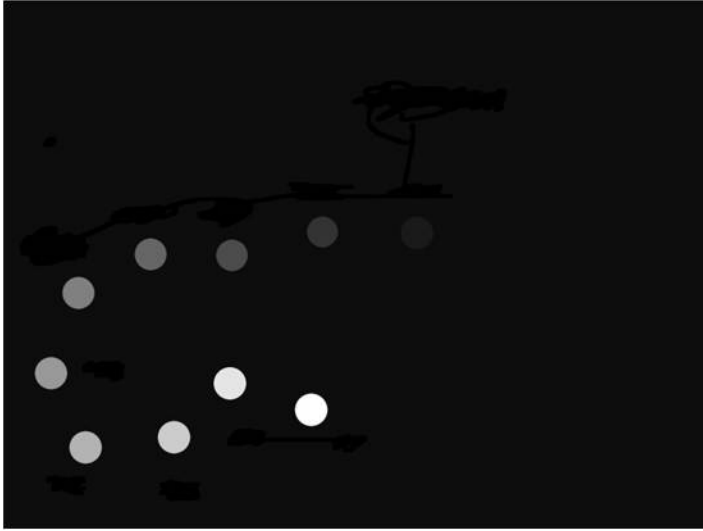


Figure D-I-1: 0 (for white) or 255 (for black) and  $8\text{Bit} = 2^8 = 256$  for different possibilities/brightness levels. If the pixel exceeds a certain threshold (which we set in the interface), it becomes white, otherwise it becomes black. The image is then transforming into 8bit  $\rightarrow$  the resulting wave is called 'M\_RGB2Gray'.

We put the mask onto the different emission channels and check the number of the cells with green or red QDs one by one. All the pictures analyzed in Igor have to be in the JPEG format and must have a filename like Name\_c0, Name\_c1 and Name\_c2 for the different channels.

```
if (Stringmatch(TopImageName1,"*c0*"))
elseif (Stringmatch(TopImageName1,"*c1*"))
elseif (Stringmatch(TopImageName1,"*c2*"))
```

Source Code D-I-2: check the name of the Image

Below I will present the two different systems which I have already mentioned above.

## 5.2. Quantification based on the transmission

### 5.2.1. Principle of the analysis

For the cells on the glass and cell culture substrates we use the transmission channel to define the position of the cells. This is the way the matrix filter sets the threshold:

```
MatrixFilter/N=(7)/P=1 median M_RGB2Gray
MatrixFilter/N=(7)/P=1 FindEdges M_RGB2Gray
MatrixFilter/N=(7)/P=1 median M_RGB2Gray
```

Source Code D-P-1: filters improve the detection of cells and at the end of a command there is always the wave which is regarded

‘Imagethreshold/I/T= (gT0)/Q root: M\_RGB2Gray’ can convert a grayscale imagematrix into a binary image; the resulting image is stored in the wave ‘M\_imagethresh’. Then, according to the principle shown in Source Code D-P-2 and Source Code D-P-3, it is modified to be used as a mask. After that step, the mask is applied to the green and red channels . This process is then repeated with three other transmission channels, although we could thus analyze as many pictures as we want.

```
ImageanalyzeParticles/M=3/A=(scaledE0)/MAXA=(scaledE0max)/W stats, root:M_imagethresh
Imagethreshold/T=40/Q root:transThresholdIncomplete
```

Source Code D-I-2: Particles (white spots) are counted that are above a certain size which you set in the interface, ' stats ' creates a number of statistics about the image (' W\_imageobjarea', 'V\_Numpaticles',.....) and ' M=3' creates the wave ' M\_particle' : Creating the mask to show the image of both the area and the boundary of the particles is done by modifying ‘Imagethreshold’.

```
duplicate/O M_Particle transThresholdIncomplete
Imagethreshold/T=40/Q root:transThresholdIncomplete
duplicate/O M_ImageThresh transThreshold

wave w_spotX
wave w_spotY
variable a
Variable V_NO=V_NumParticles
Wave W_ImageObjArea

if(V_NO<=gcellLimit)
```

Source Code D-P-3: this section here is the result of the transmission particle analysis. The additional threshold operation is just used to invert the image. The following code sets the wave's variable for the position and makes the variable for counting the number of particles. If the number of the cells is greater than the number you set in the interface it is disregarded.

```

for(a=0;a<V_NO;a=a+1)
    ImageAnalyzeParticles /L=(w_spotX[a],w_spotY[a]) mark transThreshold
    Execute "Duplicate/O M_ParticleMarker invertedsingleMask"+Num2Str(a)+" "
    Execute "Imagethreshold/T=40/Q invertedsingleMask"+Num2Str(a)+" "
    Execute "Duplicate/O M_ImageThresh SingleMask"+Num2Str(a)+" "
    Execute "NewImage SingleMask"+Num2Str(a)+" "
endfor

```

Source code D-P-4: the cycling of the images

After the analysis of C0 select all objects in the green- and red fluorescence channels, which are inside the presently selected object (cell) in the transmission channel. Cells identified as being at least  $20 \mu^2$  wide are added to the final mask as objects. The mask now contains the raw data from the original pictures to "black out" everything outside of the cells to determine them as objects. The mask is put on top of the image of the fluorescence channel. Other areas, such as noise and unwanted signals, have already been "blackened out". During the process, we add another code in order to get rid of the most common error (the overlapping of cells) as well as possible.

```

if (V_Value==1)
    if(V_greenParticles==1)
        if(V_greenParticles==V_redParticles)
            V_NumParticles=1
            V_redParticles=0
        endif
    endif
elseif (V_Value==0)
    if(V_greenParticles==1)
        if(V_greenParticles==V_redParticles)
            V_NumParticles=0
            V_redParticles=1
        endif
    endif
endif
endif

```

Source code D-P-5: deletes cells if they are labeled as both green and red, and records a green one and a red one.

The schematic description of the algorithm used and examples of images and their analysis is depicted below.

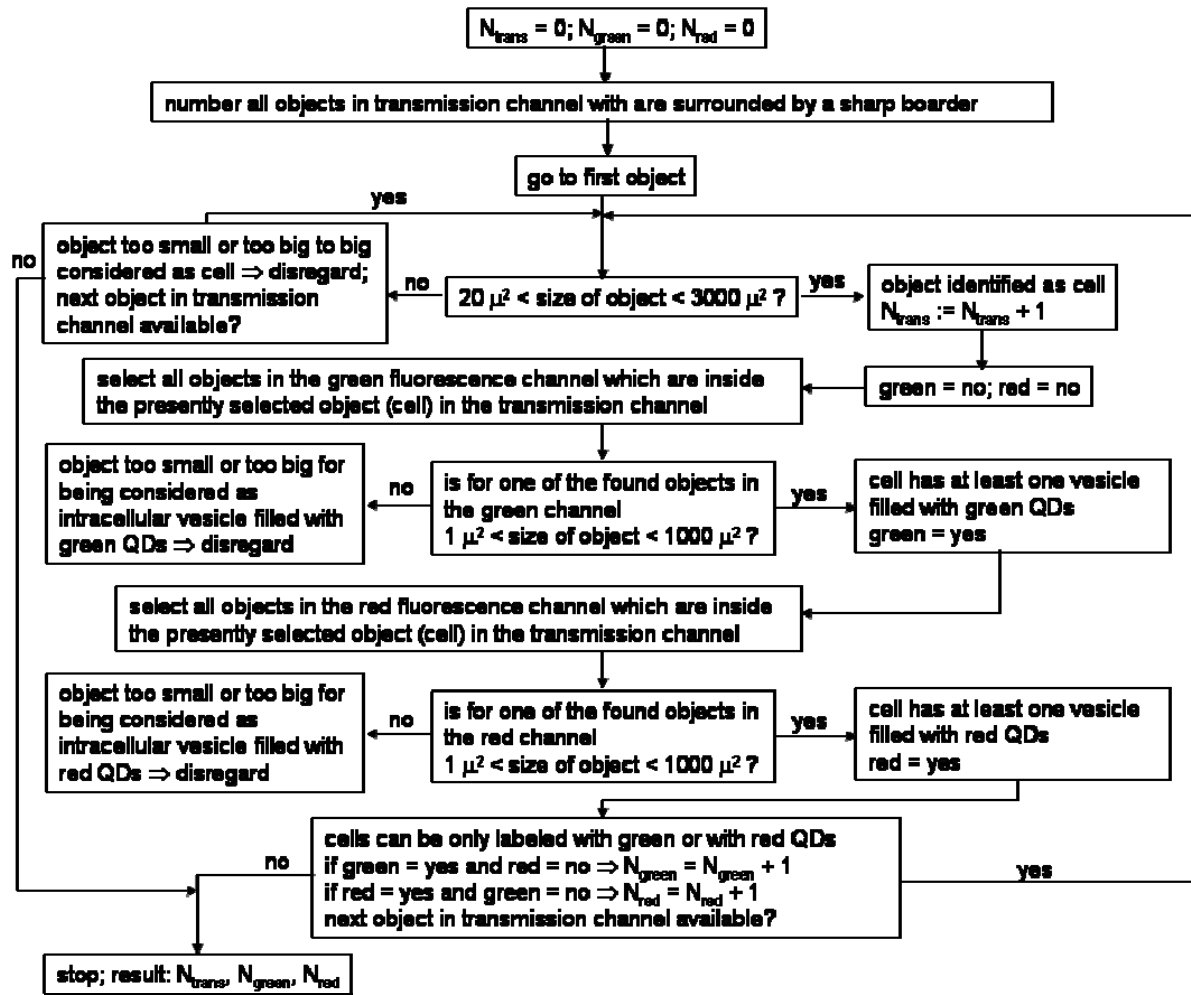


Figure D-P-1[6]: Schematic on how to analyze the images containing cells and green and red labeled cells and to count them step by step ( $N_{\text{green}}$  and  $N_{\text{red}}$ ) in different channels (trans, green and red channel).

### 5.2.2. Example of data analysis with identified cells

3T3verschRatio\_3T3\_RQD\_YQD\_X\_261008\_RQD50YQD50\_Image 24

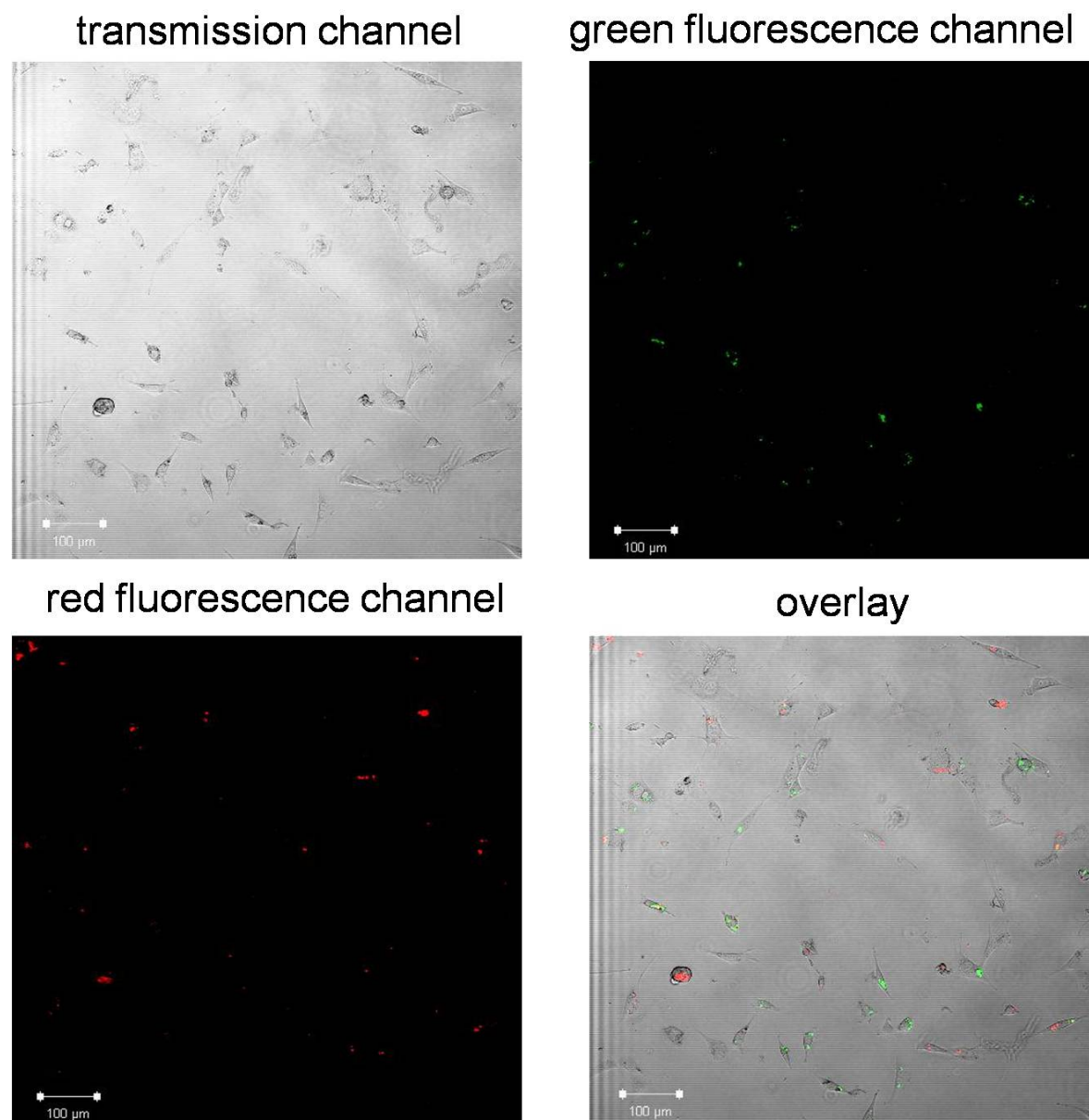


Figure D-P-E-1: The raw data of one images series is shown above. They comprise a transmission image and images obtain in the green and red fluorescence channel. In addition, an overlay image of all channels is shown.

green fluorescence channel

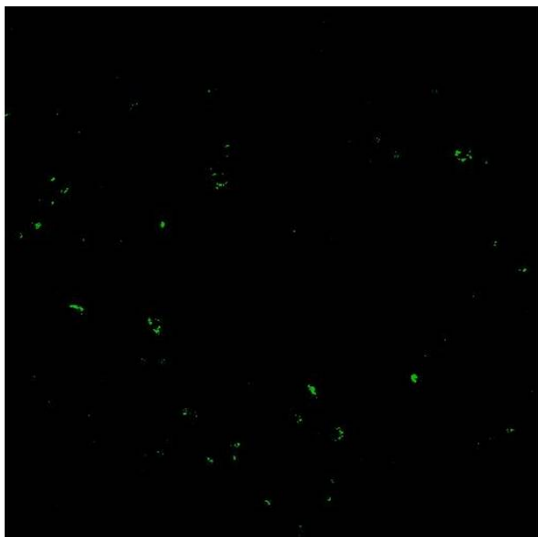
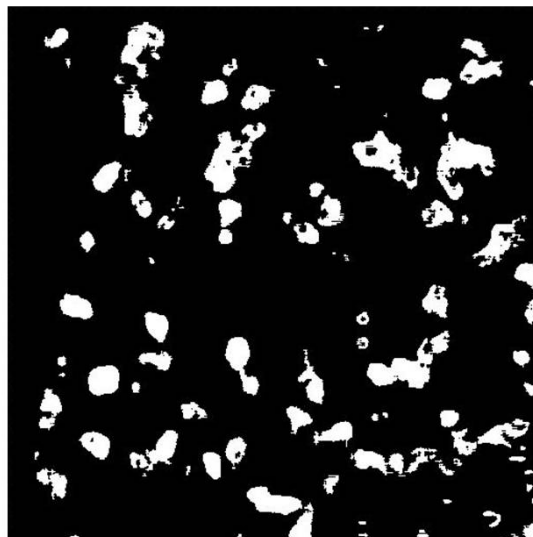
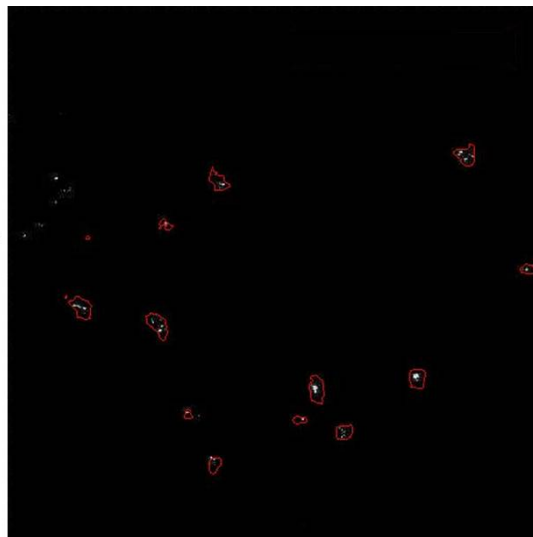
mask for objects which  
are identified as cellsmask put on top of green  
fluorescence channelobjects identified as  
green fluorescent QDs

Figure D-P-E-2: The mask created from the transmission channel is put on top of the green fluorescence channel (from the raw data as shown in Figure D-P-E-1). In this way, fluorescence in the regions outside of the borders of the cells is "blacked-out". Fluorescence in the green channel above a certain threshold at the regions attributed to cells regions is attributed to intracellular vesicles filled with green fluorescence QDs.



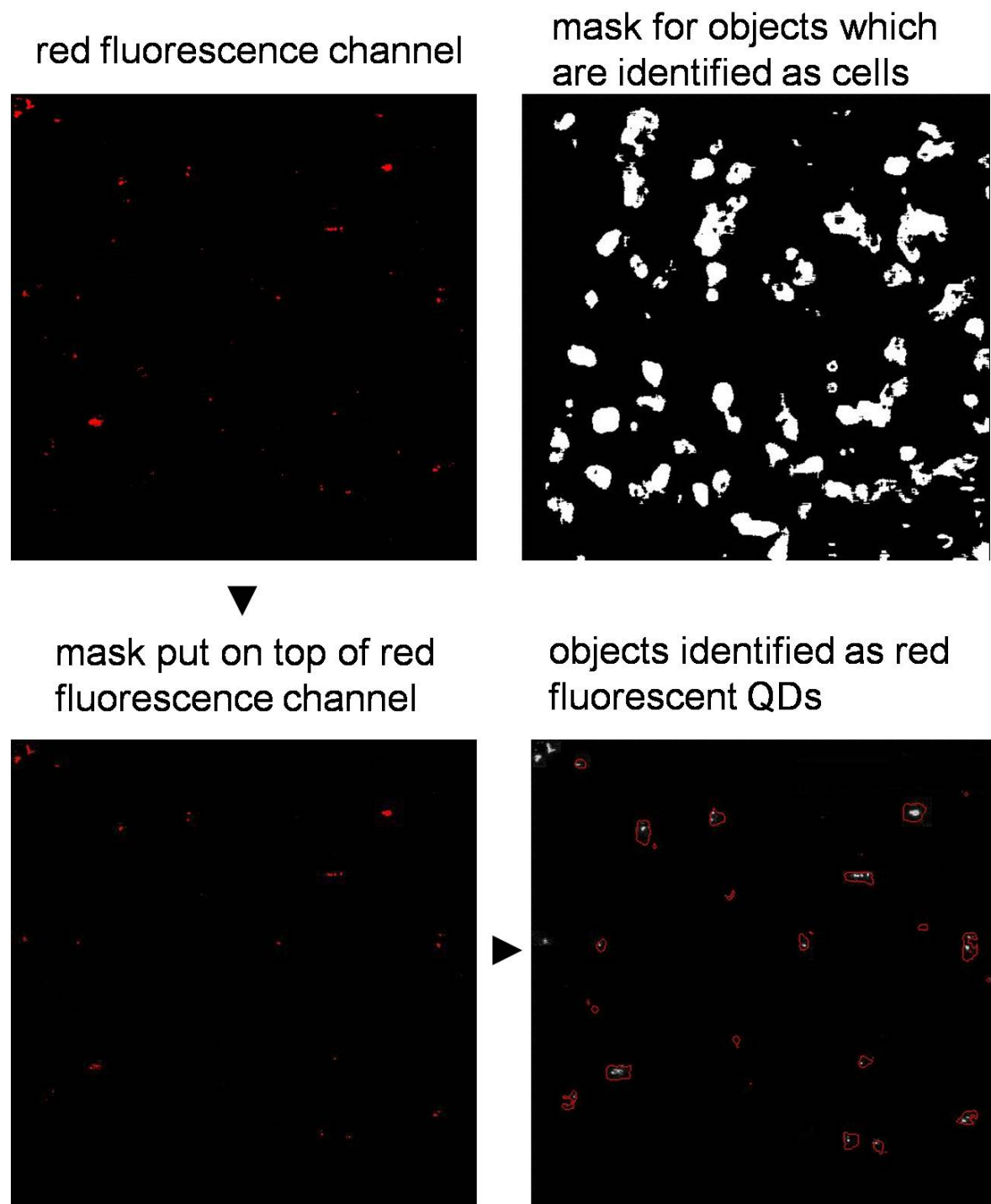


Figure D-P-E-3: The mask is put on top of the red fluorescence channel (from the raw data as shown in Figure D-P-E-1); the process is identical to the process applied to the green fluorescence channel.

## 5.3. Quantification based on DAPI

### 5.3.1. Principle of the analysis

Because we need to use different patterns and substrates, such as glass and gold, which have different structures (diamond-shaped shaft patterns, line patterns etc.) with sizes of 10 nm to 1 mm, as well as different chemicals (hexadecanethiol, ethylene glycol, perfluorothiol). This creates different gradients on the same types of glass. The borders between the different areas of the surface coatings gave contrast and could not be distinguished from cells with standard image analysis programs. In order to get rid of the problem we develop the DAPI-system.

Actually the DAPI is 4', 6-Diamidino-2-phenylindole, dictate, which is a common blue fluorescence dye used for staining the nuclei of live cells. After noise was filtered out, the cells were then identified by their stained nuclei in the blue fluorescence channel. The dye covers the entire object and then moves to the nuclear and perinuclear regions. This channel was used to create a digital mask, which is transparent where there are nuclei and perinuclear regions and black in all other positions. This digital mask is then applied to the green and red fluorescence channel images. Thus, the fluorescence is still only in the nucleus and the position of the nucleus is determined. The nuclei of the cells in the blue fluorescence channel are surrounded by a sharp border. Where intracellular vesicles are located around the nucleus, they must be no more than 4 $\mu$ m away from the object (width of the core area) to be identified as a new object (nucleus + core area of the nucleus). We then put the digital mask on the top of the fluorescence channel and select all objects which are inside the previously identified areas (nucleus + core area of the nucleus) in the blue channel. This process can be repeated to count the green and red QDs on other images. We also use the 'ImageHistModification/O M\_RGB2Gray' to improve the quality of the image of the fluorescence channel. The process is similar to the threshold-based technique described above.

The modification of the technique for identifying the location of cells by their blue fluorescence and the solution to the problem whereby cells are labeled as both green and red will now be described.

```
Wave W_ImageObjArea

ImageAnalyzeParticles /L=(w_spotX[a],w_spotY[a]) mark transThreshold
Execute "Duplicate/O M_ParticleMarker invertedsingleMask"+Num2Str(a)+" "
Execute "Imagethreshold/T=40/Q invertedsingleMask"+Num2Str(a)+" "
Execute "Duplicate/O M_ImageThresh SingleMask"+Num2Str(a)+" "
Execute "NewImage SingleMask"+Num2Str(a)+" "
```

Source code D-P-5: Only one single cell is examined. "w\_spotX[a], w\_spotY[a]" locates the position of the cell with its spot of blue fluorescence. They are all as mark in the same mask picture.

```
if (V_Value==1)
if(V_greenParticles==1)
if(V_greenParticles==V_redParticles)
V_greenParticles=1
V_redParticles=0
endif
endif
elseif (V_Value==0)
if(V_greenParticles==1)
if(V_greenParticles==V_redParticles)
V_greenParticles=0
V_redParticles=1
endif
endif
endif
if (V_Value==1)
if(V_redParticles==0)
if(V_greenParticles==V_redParticles)
V_greenParticles=0
V_redParticles=1
endif
endif
elseif (V_Value==0)
if(V_redParticles==1)
if(V_greenParticles==V_redParticles)
V_greenParticles=1
V_redParticles=0
endif
endif
endif
endif
```

Source code D-P-5: If cells are labeled with green as well as with red QDs, we can choose which should be counted.

That is very useful when two cells overlap, as this causes one cell to be labeled as both green and red (see D-P-5).

The schematic description of the used algorithm and examples for images and their analysis are depicted below.

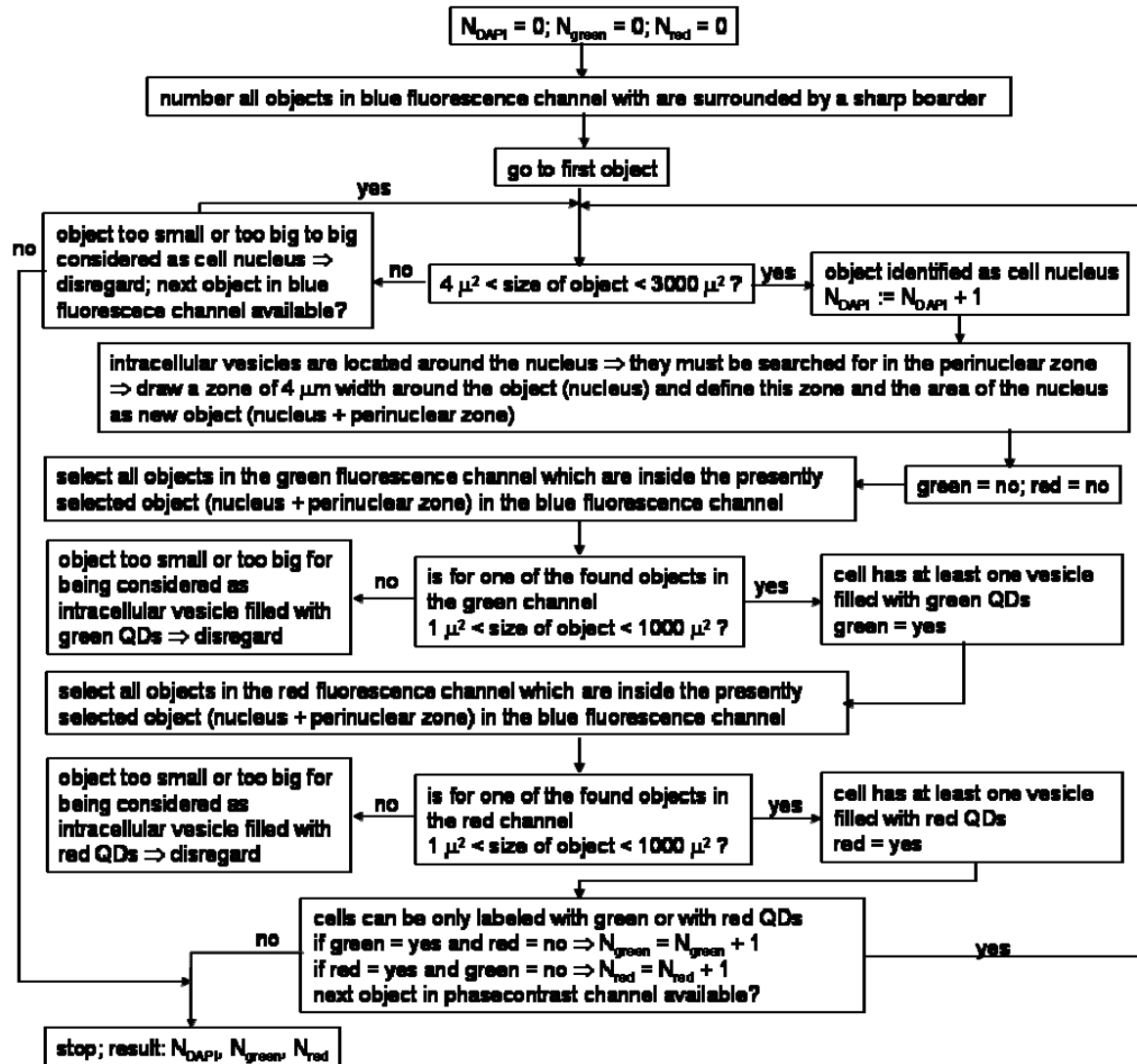


Figure D-D-2[6]: Schematic on how to analyze the image of the cells with  $N_{DAPI}$  and green and red labeled cells and then count them, step by step, ( $N_{green}$  and  $N_{red}$ ) in the different channels (DAPI, green and red channel).

### 5.3.2. Example for data analysis with identified nuclei

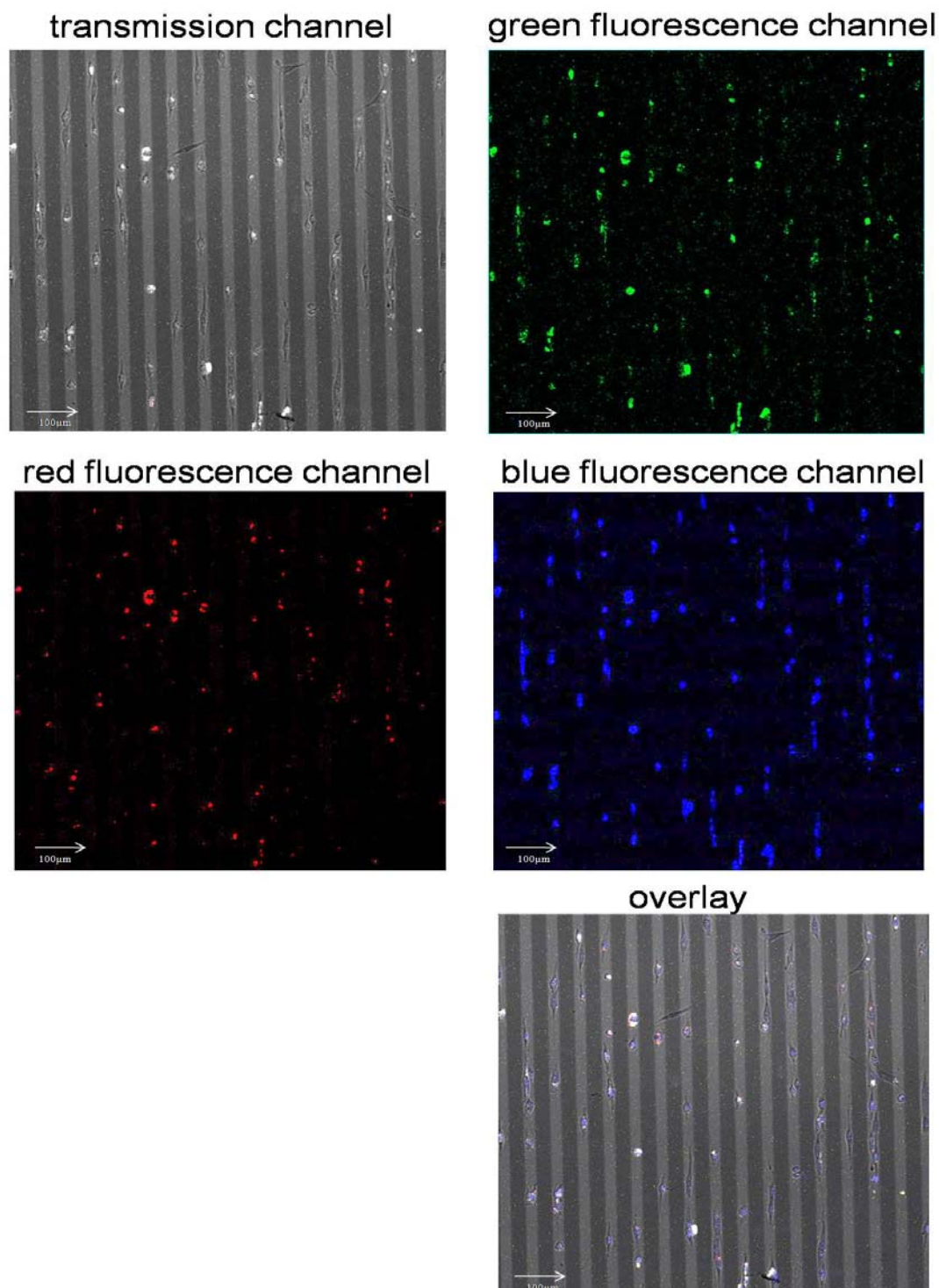
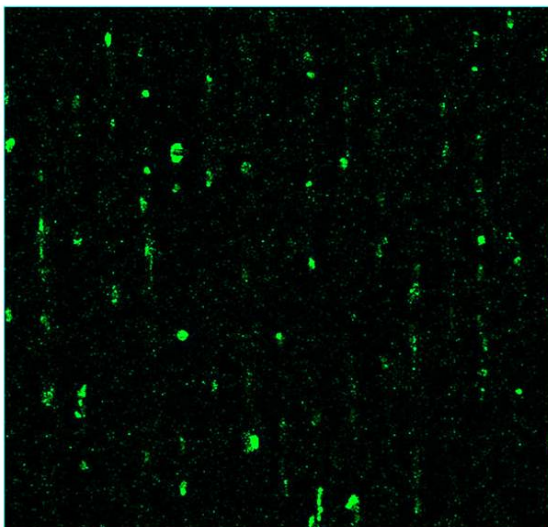
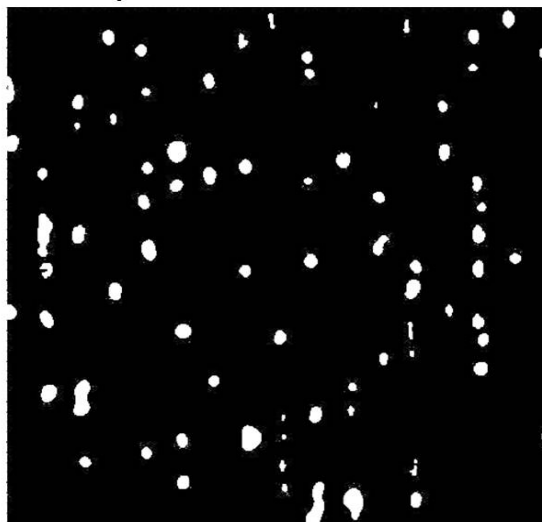


Figure D-D-E-1: The raw data of one images series is shown above. They comprise a transmission image and images obtain in the green and red fluorescence channel. In addition, an overlay image of all channels is shown.

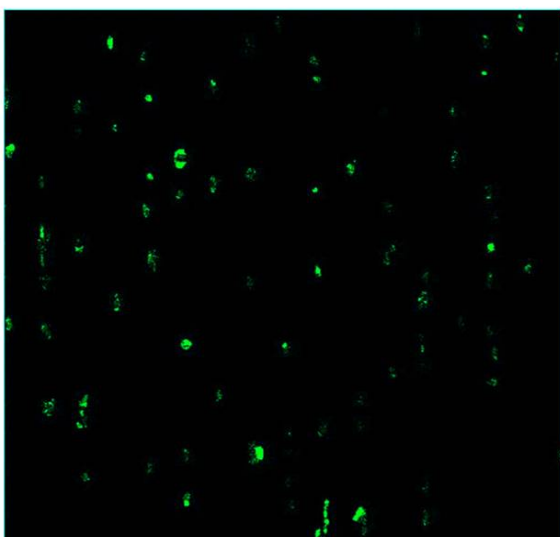
green fluorescence channel



mask for objects which  
are identified as nucleus  
and perinuclear zone



mask put on top of green  
fluorescence channel



objects identified as  
green fluorescent QDs

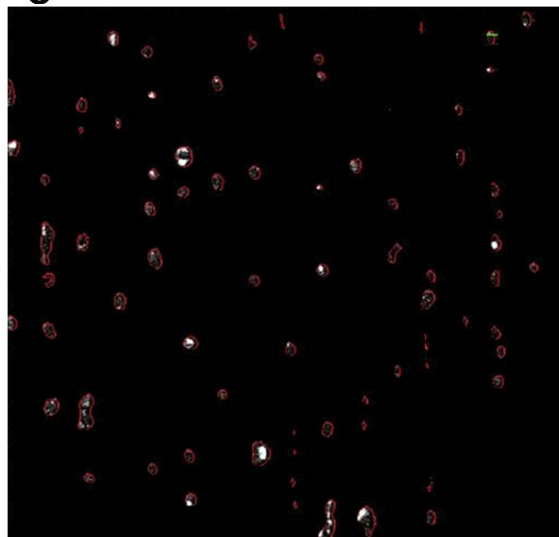
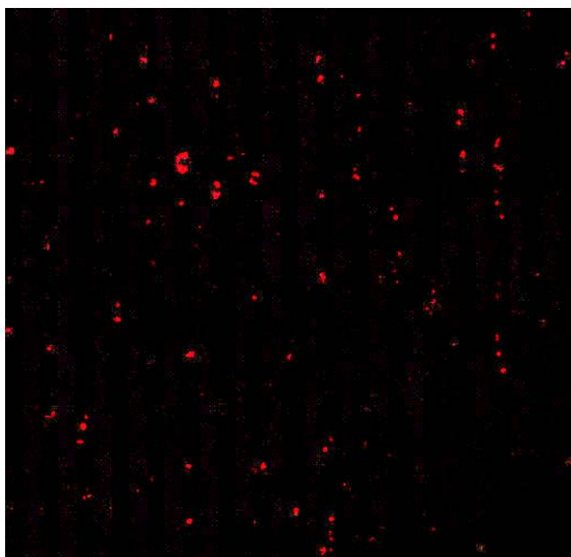


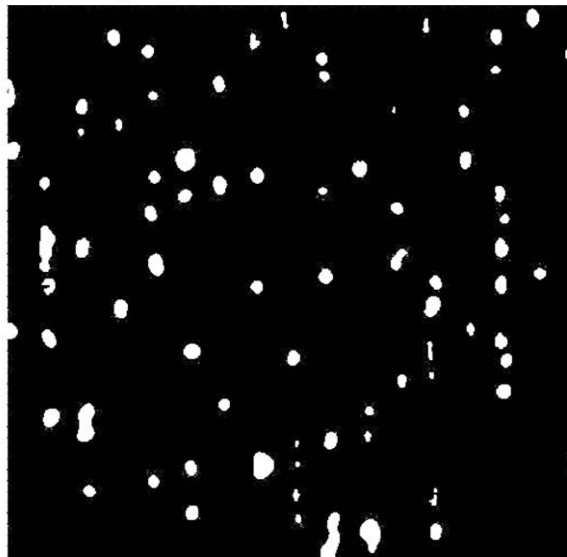
Figure D-D-E-2: The mask created from the DAPI channel is put on top of the green fluorescence channel (from the raw data as shown in Figure D-D-E-1). In this way, fluorescence in the regions outside of the borders of the cells is "blacked-out". Fluorescence in the green channel above a certain threshold at the regions attributed to cells regions is attributed to intracellular vesicles filled with green fluorescence QDs.



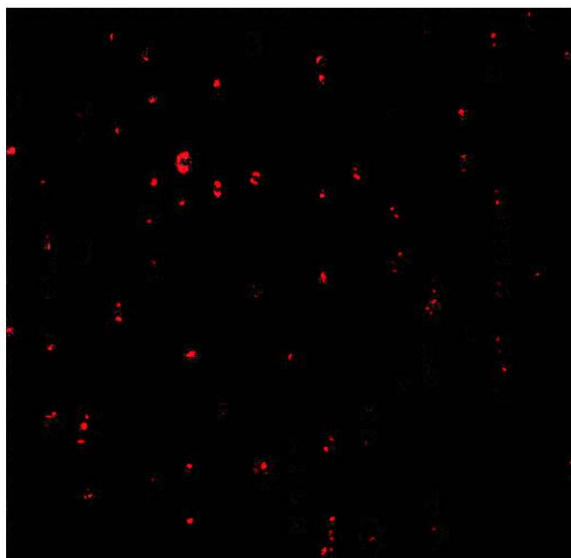
red fluorescence channel



mask for objects which  
are identified as nucleus  
and perinuclear zone



mask put on top of red  
fluorescence channel



objects identified as red  
fluorescent QDs

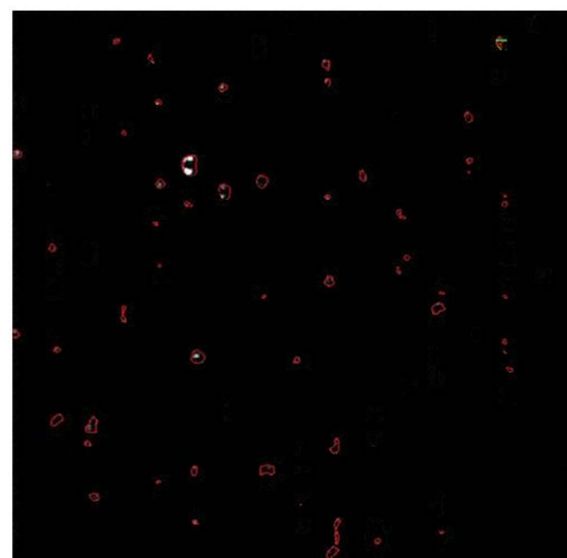


Figure D-D-E-3: The mask is put on top of the red fluorescence channel (from the raw data as shown in (Figure D-D-E-1)); the process is identical to the process applied to the green fluorescence channel.

## 5.4. Software module

After compiling the advanced procedure files we can open the directory containing the image of the cells. We then open the menu shown in the Figure D-S-1, and then we choose FullRatioAnalysis, which opens two windows where we can determine the location of the cell image file (see Figure D-S-2) and set the directory in which the results will be saved. After choosing, we can see the control panel, in which we can define the settings and conditions under which the program will analyze the images (e.g. thresholds for brightness levels). In addition, there are also checkboxes for switching off the QDs, setting the scaling factor [4] and limiting the maximum number of cells [4] (see Figure D-S-3). We can then run the right method according to what we need.

### Setting up the Igor software

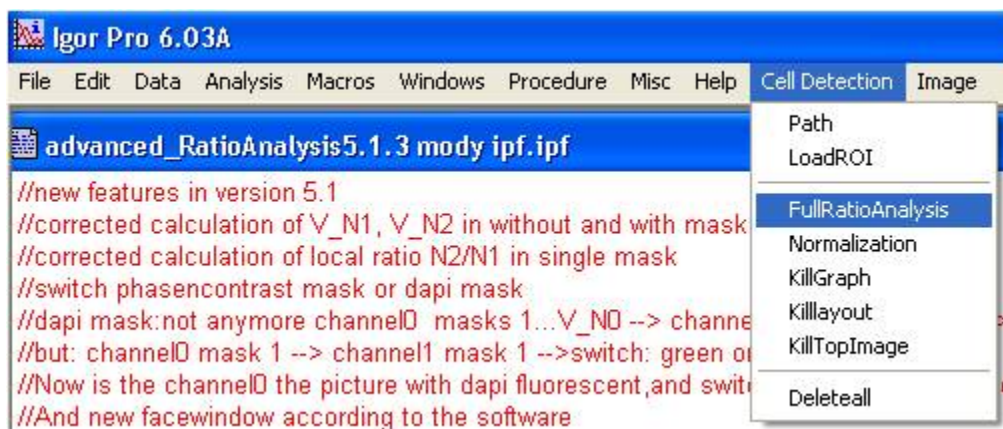


Figure D-S-1: This is what the window looks like after compiling the advanced ipf

The window for determining the location of the cell image file (followed by the window for setting the directory for saving the results data):

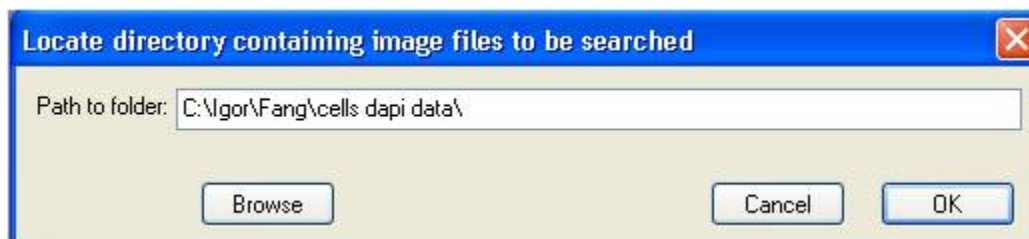




Figure D-S-2: 1. Determining the location of the cell image file; 2. Setting the directory for the results data (not shown).

## The interface of the Igor program

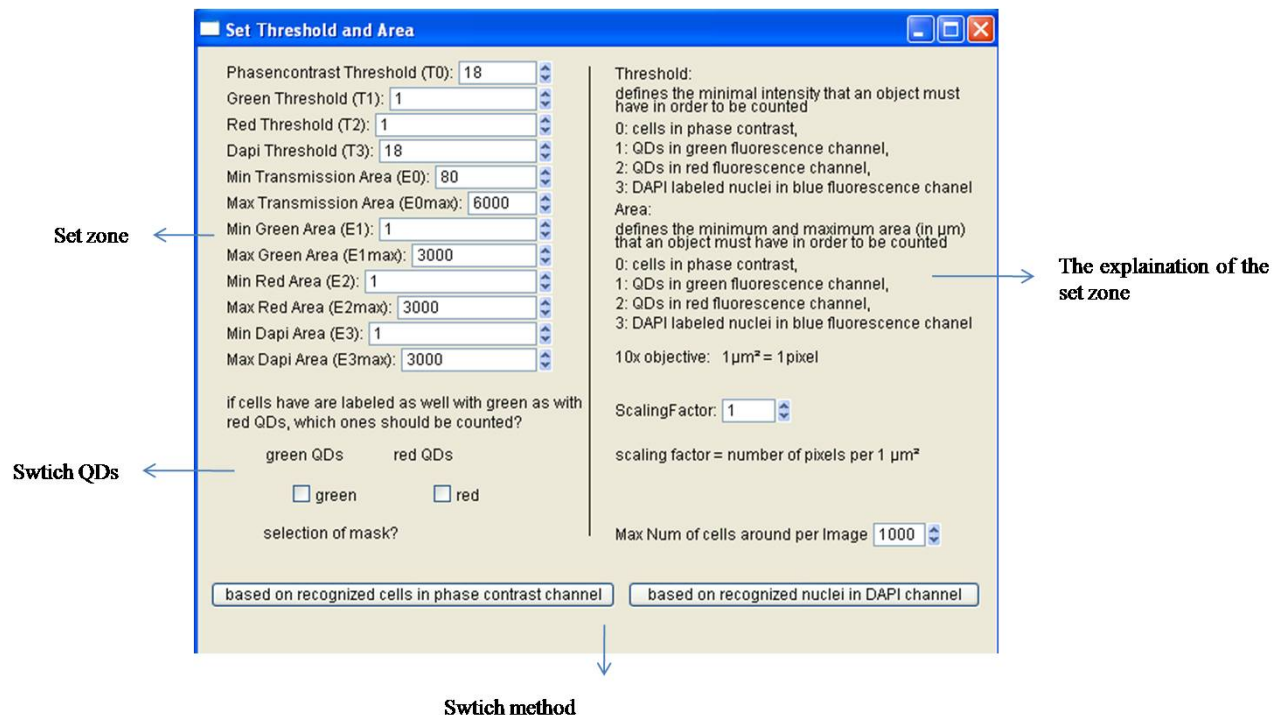


Figure D-S-3: This window is the main control panel, where you define all the settings for the analysis.

After the analysis is complete, some of the results are displayed in a window which pops up immediately, which are shown in the form of a table, along with a summary of the results and images. From this table we can get the results of N0 or N3, N1 and N2; the summary of the results records the settings that were set in the control panel and displays the method used. In the final image, the program frames the locations of the QDs in red.

## Analysis Results with based on dapi mask

## Settings

Threshold for Dapi, Green and Red: 1 , 1 , 1

Minimum Particle Area for Dapi, Green and Red: 2 , 1, 1

Max Area: 3000 , 3000, 3000

Scaling Factor: 1

Upper Cell Limit: 1000

GreenParticles: on

Point	N3	N1	N2
0.00	49.00	23.00	41.00
1.00	38.00	16.00	25.00
2.00	48.00	20.00	36.00
3.00	56.00	20.00	28.00
4.00	33.00	8.00	13.00
5.00			

RedParticles: off

Version 5.1

Figure D-S-4: An example of a table and a summary of results.

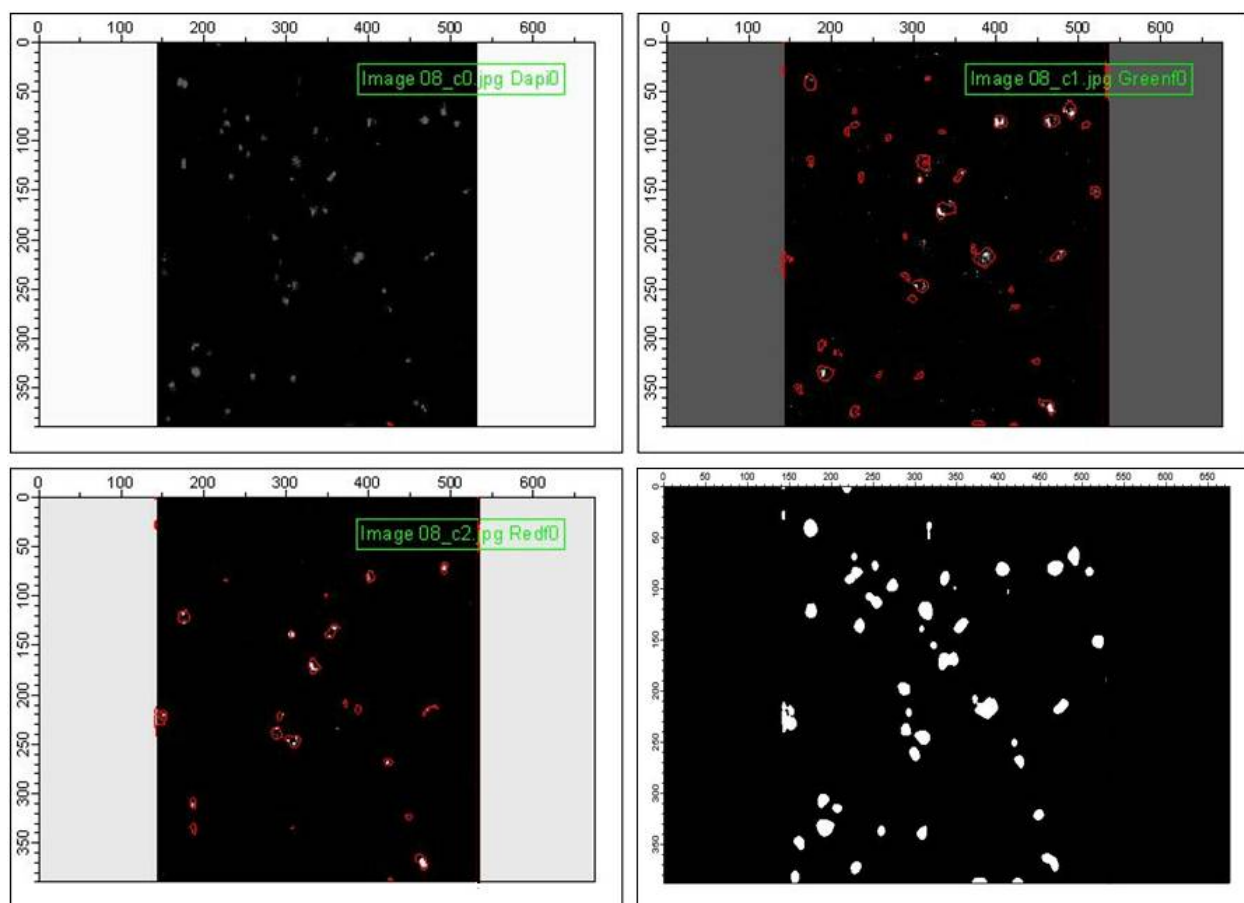


Figure D-S-4: An example of a resulting image with the QDs framed in red.

## 6. Result and discussion

Because the size of the structures of the different substrates influences the attachment of the cells, we use different substrates to change the spacing between the adhesive and non-adhesive parts. The cells adjust their morphology to the substrate, so we use any two different types of cells, which are labeled with two different QD colors for identifying the respective cells in coculture, after which we observe them, calculate their number and record the results in a statistic. After we get the statistic, which displays the number of the  $N_{\text{Trans}}/N_{\text{DAPI}}$ ,  $N_{\text{green}}$  and  $N_{\text{red}}$ , we then analyze them with the formula in Figure B-1. Figure R-1 shows the different distances of the lines on the gold substrates.

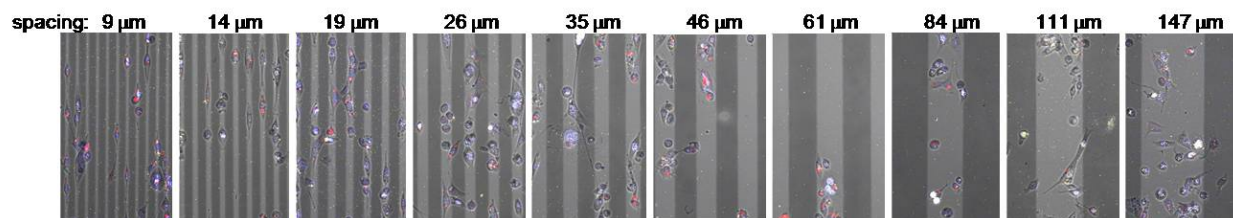


Figure R-1: Increases in the spacing of pattern size.

As an example using the two cells 3T3 (with green QDs) and A549 (with red QDs) is shown below. By calculating the number of cells in the adhesive part (the spacing), we have found a relatively easy way to quantify cell adhesion within cell-coculture.

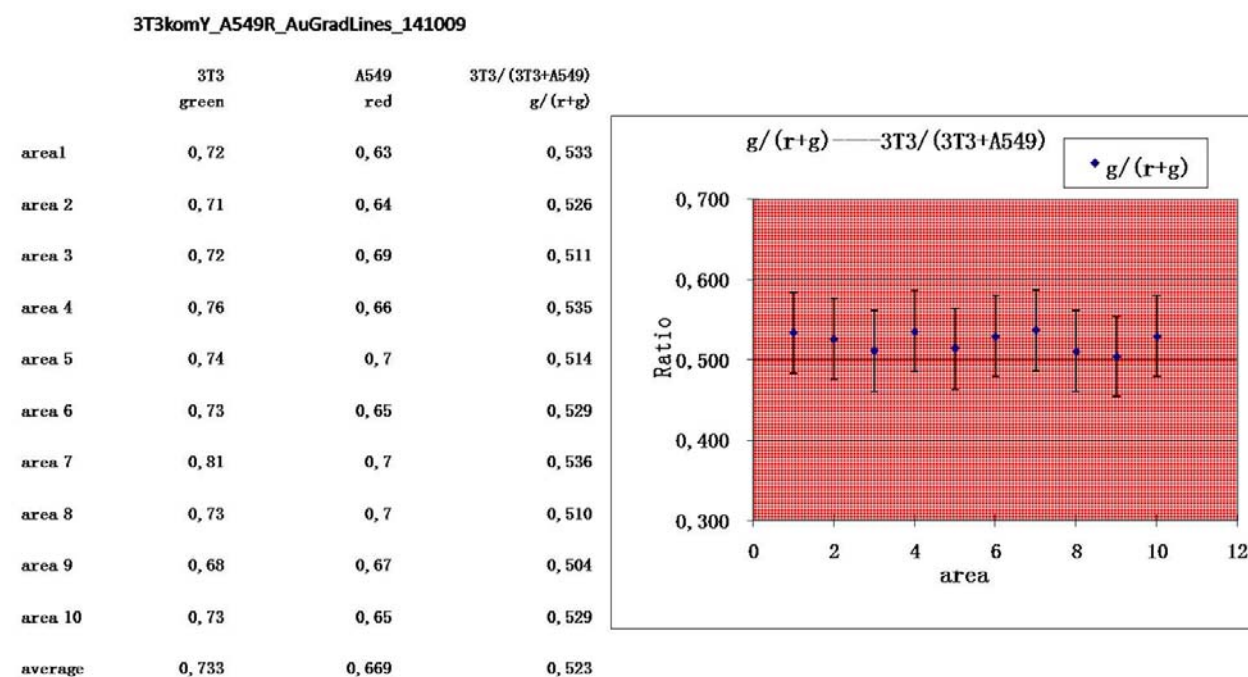


Figure R-2: The data shows that cell 3T3 can adapt to rather narrow and long morphology, because they appear in a higher ratio in the narrowly spaced gold lines, whereas the ratio of A549 cells to 3T3 cells becomes closer to 1 as you increase the spacing of the gold.

## 7. Outlook

Of course, no program is perfect and they always need to be improved over time. For example, Igor Pro can only count locations in which there is a certain amount of fluorescence as ROI. In addition, we can not always count labeled cells very exactly, because they are sometimes too close each other. These could potentially influence our results. As a result, we need to create an entirely new method of counting QDs by determining their intensity, because their intensity depends on how many of them are in a particular batch. As a result, we could make a diagram showing the relationship between the intensity and the number of the QDs, which would allow us to count them exactly. The following description will show how this could be done.

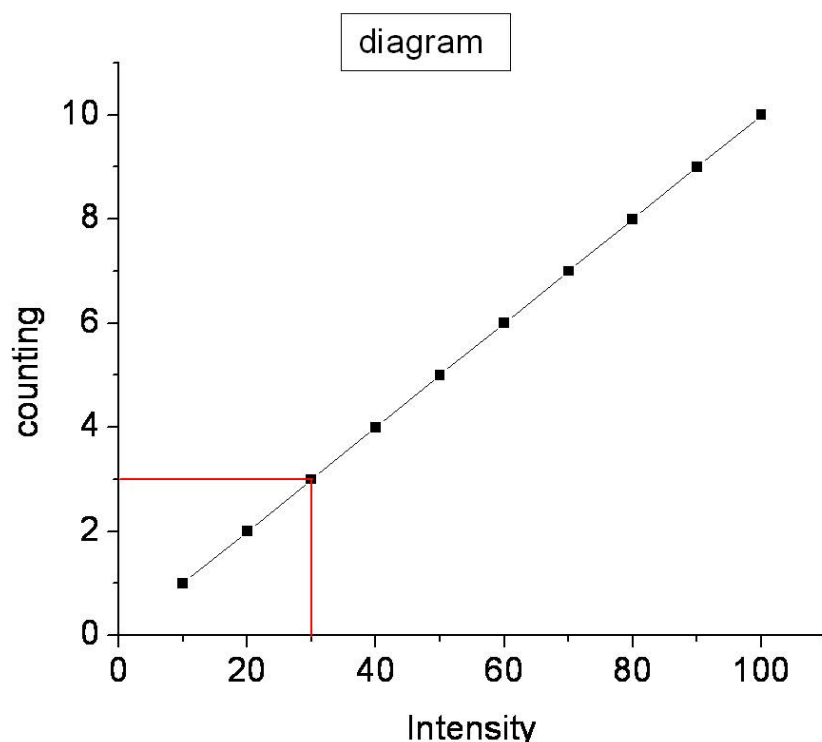


Figure N-1: If we can determine the intensity, we could then know the exact number of QDs. This is a rough diagram of how the intensity and the number of QDs would relate to each other.

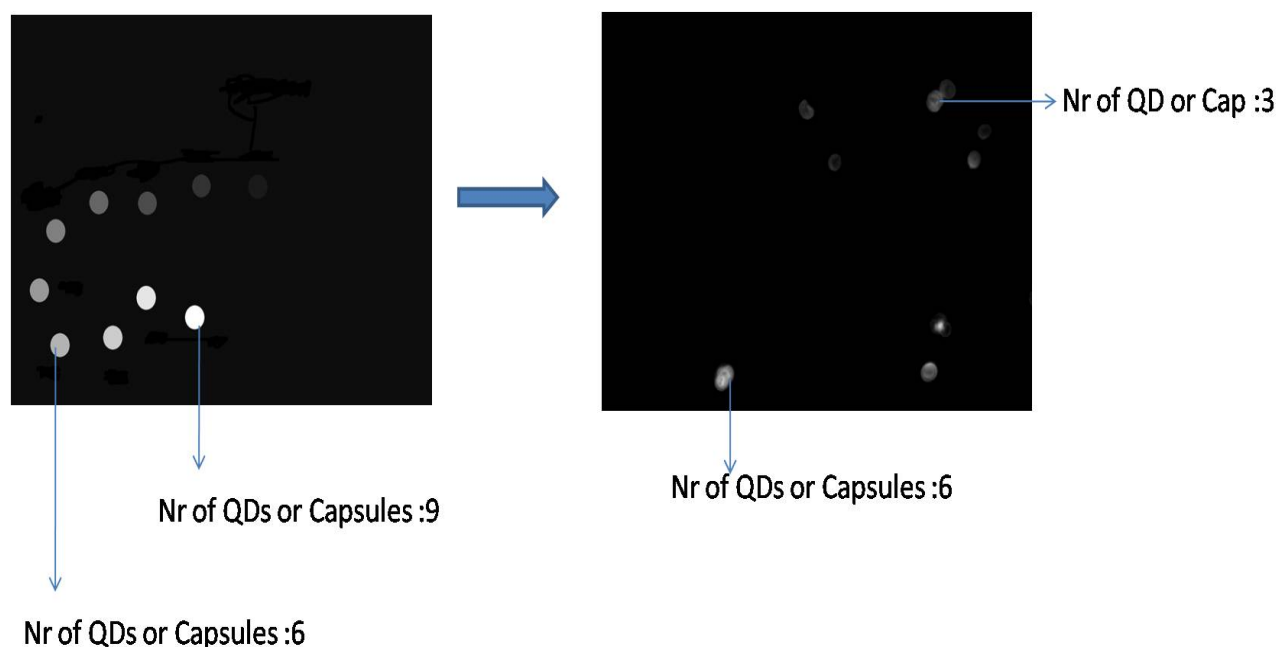


Figure N-2: We could measure the contrast between the intensity around the objects (left picture) and the “blacked out” area. By measuring the intensity, we could calculate the exact number of QDs.

**Conclusion:** The presented method is one of the best ways for quantifying cell adhesion within cell-coculture. It improves the accuracy of the assay and makes counting the number of objects much easier. This software can analyze particles in images and is specially adapted for the assay of cells labeled with QDs, but we should modify the software in order to adapt it to more diverse types’ analysis.

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## 9. References

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- [3] W.J.Parak, T.Pellegrino, C.Plank et al.(2005). Labelling of cells with quantum dots. “*Nanotechnology* 16: R5-R25.
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